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AWARD NUMBER: DAMD17-01-1-0772

TITLE: Improving Blood Monitoring of Enzymes as Biomarkers of Risk from Anticholinergic Pesticides and Chemical Warfare Agents

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REPORT DATE: October 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-10-2006	Annual	24 Sep 2005 – 23 Sep 2006

4. TITLE AND SUBTITLE

Improving Blood Monitoring of Enzymes as Biomarkers of Risk from Anticholinergic Pesticides and Chemical Warfare Agents

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0772

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California
Davis, California 95616-8671

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Blood biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare agents and to establish whether some personnel are at greater risk than others from exposure. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine. CHPPM (US Army Center for Health Promotion and Preventive Medicine) uses a slower delta pH method based on that of Michel to monitor 16,000 DOD personnel each year. Two different approaches of ours yielded conversion factors for expressing delta pH AChE in terms of Ellman assay units. We also converted the normal range of AChE activities from the CHPPM delta pH assay to Ellman units generating important benchmarks for clinical laboratory determinations in the absence of baseline data. Future work includes determining conversion factors for the Test Mate cholinesterase measurements to the delta pH and Ellman methods, and examining the feasibility of monitoring serum BChE and PON1 activities in collaboration with the CRL laboratory of CHPPM.

15. SUBJECT TERMS
Blood biomarkers, chemical warfare agents, pesticides, cholinesterases

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

There is a need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) levels to provide early warning of exposures due to the intensive use of pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents. The colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al*., 1961) is used by many clinical and research laboratories. A related ChE method has been developed at the WRAIR laboratory (Feaster, *et al*., 2001). A slower delta pH method, based on that of Michel (1949), is used to monitor the erythrocyte (RBC) acetylcholinesterase (AChE) of greater than 15,000 DOD personnel involved with chemical nerve agent and demilitarization operations each year. The assays are conducted by the Cholinesterase Reference Laboratory (CRL) at the US Army Center for Health Promotion and Preventive Medicine (CHPPM). Although pH assays are reliable and have low variability, they are not readily adaptable for kinetic analysis, automation or field use.

One goal of this project was to establish a conversion factor between the pH and colorimetric assays applicable to monitoring studies and field tests. Another goal was to provide conversion factors for the portable Test-Mate kit manufactured by EQM, Inc., purchased by the Army for "field use". We showed that the current model is not adequate under "strict" field conditions (Oliviera *et al*., 2002). Plans agreed to by the manufacturer were to produce a new model with improved assay parameters and adjustments.

Another issue was that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered plasma butyrylcholinesterase (BuChE), a scavenger of antiChE agents, may put individuals at increased risk to OP and CB agents (reviewed by Wilson, 1999). Paraoxonase (PON1) hydrolyses nerve agents (soman, sarin and VX) and the active oxon metabolites of widely used OP pesticides (diazinon and chlorpyrifos) in addition to paraoxon (Costa, *et al*., 2005a). PON1 has been reported to be reduced in a cohort of veterans suffering from "Gulf War Syndrome" (Haley *et al.*, 1999). PON1 levels can also be modulated by lifestyle factors, such as diet, smoking and alcohol (Costa, *et al*., 2005b).There is evidence that low levels of BChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991). Following completion of the cholinesterase tasks, plans were to investigate the feasibility of screening for lowered BuChE and PON1 activities in CHPPM blood samples.

BODY

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample collection

Blood was obtained from volunteers at UCD under an approved Human Subjects Protocol. Blood was collected in EDTA vacutainers and kept on ice. Within 4 hours of

collection, RBCs were separated by centrifugation at 1000 x g for 15 minutes (plasma discarded). The RBCs were washed with phosphate buffered saline (PBS) and then recentrifuged. The separated RBCs were stored at 4^{o}C.

Ellman Cholinesterase Assay

RBCs were diluted 1/50 in 0.5% Triton X-100, 0.1 M sodium phosphate buffer, pH 8. AChE activity was measured using a modified colorimetric method of Ellman *et al*. (1961) in 96 well plates at 25°C. The final concentrations of the substrate acetylthiocholine and the color reagent dithiobisnitrobenzoate (DTNB) were 1 and 0.24 mM respectively. Activity was reported as umol/min/mL RBC.

Delta pH Cholinesterase Assay

Delta pH measurements determined at CRL and UCD were performed according to Standard Operating Procedure # CRL40-2.7 provided by CRL. A 200 ul aliquot of RBCs was added to 4 ml of assay buffer (13 mM sodium barbital, 3 mM potassium phosphate monobasic, 510 mM sodium chloride, and 0.012% (w/v) saponin, pH 8.05). An initial pH measurement was recorded prior to adding acetylcholine bromide (10 mM final concentration), followed by a final pH measurement seventeen minutes later. The pH change of a substrate blank (no RBCs present) averaged 0.05 $\pm$ 0.02 delta pH/hr (n = 9). Assays were carried out at 25°C. Results were expressed as delta pH/hour.

Test-Mate OP Kit Assay

Test-Mate measurements were made following the instructions for the FDA approved kit. Centrifuged RBCs were resuspended in an equal volume of PBS. Ten ul of RBC mixture were added to the assay vial with an automatic pipetter (not with the provided glass capillary). The kit's reagent mixture was added to the vial and the assay carried out in the Model 400 Test-Mate ChE. Reagent concentrations are 1 mM ATCh and 3 mM DTNB. Activity was reported as umol/min/g Hb.

Task One. Conduct a careful comparison of the Ellman assay performed under optimum conditions and the DOD pH assay to examine the variability and reliability of both assays, to establish baseline values and to generate conversion factors to enable comparisons between them and other proposed or commercially available assays.

The main objectives of Task One have been completed as detailed in previous annual reports. Conversion factors were estimated by 2 different paradigms. Measurements with the CHPPM delta pH assay and the UCD Ellman assay, in split RBC samples collected by CHPPM yielded: Ellman = 15.0(delta pH/hr) - 3.06. Assays of diisopropylfluoro-phosphate (DFP) inhibited RBCs yielded Ellman = 10.5(delta pH/hr) + 0.13.

We will complete this task by using a variety of OPs (possibilities include XGB, diazinon-oxon, chlorpyrifos-oxon, paraoxon) to inhibit volunteer blood samples. These will be assayed with both methods as a check of our conversion equations. The agents are on hand and facilities have complete UCD and USAMRICD approvals.

Task Two. Test the stability and usability of a red blood cell ghost standard suitable for clinical standardizations.

Task Two has been completed and the results have been published **(**Arrieta *et al*., 2003). The activity of the earlier ghost RBC (gRBC) preparations was too low to measure with the delta pH method. We have increased the activity level in new preparations and are re-examining the issue. The ghost RBC standard is included routinely in each Ellman microplate assay conducted at UCD.

Task Three. Conduct experiments with a specially designed Test-Mate Kit with an uncorrected read out to establish the conditions for an optimum assay and construct conversion factors to harmonize its results with clinical laboratory assays.

Unfortunately, such a specialized Test-Mate was never made available to us by EQM. Instead we are utilizing the Model 400 (in use by the military), to establish a conversion factor with the delta pH method. Results to date are presented.

Blood from 5 volunteers was inhibited by a range of DFP concentrations and assayed by three cholinesterase methods: delta pH, Test-Mate and Ellman. The comparison of delta pH and Test-Mate results is shown in Figure 1. The line estimate of a conversion factor is Test-Mate (umol/min/g Hb) = 0.156 x (delta pH/hr) + 0.15, with a correlation coefficient (r^2) of 0.99.

When this conversion factor was combined with the 991 delta pH values from CHPPM used earlier in the project, we obtained a range of Test-Mate values of 18.4 to 47.2 umol/min/g Hb. The mean value was 28.6 with a standard deviation of 2.90 umol/min/g Hb.

The comparison between the Test-Mate and Ellman assays is shown in Figure 2, and between the delta pH and Ellman assays in Figure 3. The line estimates are Test-Mate (umol/min/g Hb) = 0.409 x (Ellman umol/min/mL) – 0.33, and delta pH/hr = 0.064 (Ellman umol/min/mL) + 0.09, respectively. The r^2s are 0.93 and 0.92.

The higher activities appear to deviate from the linear regression in Figures 2 and 3. Another estimate of the regressions were made, splitting the data into groups that are below or above an Ellman activity of 8.0 umol/min/mL. These are presented in Figures 4 and 5. The r^2 values of the data below 8 were higher than the values for the complete data (0.94 and 0.94). The estimates for data above 8 had poor correlations (r^2 values of 0.37 and 0.36) and much flatter slopes.

Task Four. Explore the feasibility of incorporating BuChE variant and PON1 polymorphisms into a screen of workers for whom blood ChE baselines are required using a selected set of DOD personnel.

We are in contact with Captain Gull, the director of CHPPM, and Major Lefkowitz, the former manager, to determine if such a screen could be practically implemented. One

approach to screening for BChE and PON1 is to focus on activity levels of the two enzymes using butyrylthiocholine and the Ellman assay for BChE and the colorimetric two substrate (diazoxon and paraoxon) PON1 assay (Richter, *et al*., 2004, Costa, *et al*., 2005a). This would require procedural changes in how blood is collected and handled at CHPPM before any new assays could be carried out. Separated plasma would need to be collected in addition to the RBCs. EDTA collection tubes could not be used due to interference with the proposed PON1 assay. Additional equipment may be necessary at the CRL to carry out these assays.

KEY RESEARCH ACCOMPLISHMENTS TO DATE

1. A normal range for human RBC AChE with CHPPM data was established.
2. Two conversion factors for AChE activities between CHPPM delta pH data and Ellman values were obtained.
3. Conversion factors were used to generate a normal range of human RBC values in Ellman units, useful for clinical laboratories.
4. A conversion factor for AChE activities between the CHPPM delta pH and Test-Mate assays, methods currently used by the military in the laboratory and in the field, was obtained.
5. The conversion factors were used to generate a normal range of human RBC values in Test-Mate units, appropriate for military units using the Test-Mate kit.

REPORTABLE OUTCOMES

Arrieta DE, SA McCurdy, JD Henderson, LJ Lefkowitz, RE Reitstetter and BW Wilson. 2004. Normal Range of RBC Cholinesterases in a DOD Monitoring Program. To be submitted.

Arrieta DE, VM Nihart, JD Henderson, A Ramirez, LJ Lefkowitz and BW Wilson. Monitoring Cholinesterases: Progress Toward a Normal Human Range and Conversion Factors. Presented at the Bioscience Review; June 4-9, 2006; Hunt Valley, Maryland.

Wilson BW, VM Nihart, JD Henderson, A Ramirez and DE Arrieta. Monitoring Cholinesterases: An Example of Translational Research. Presented at the 45th Annual Meeting of the Society of Toxicology; March 5-9, 2006; San Diego, California.

Wilson BW, JD Henderson and DE Arrieta. Cholinesterase Assays as Indicators of Exposure. Presented at 229th American Chemical Society National Meeting; March 13-17, 2005; San Diego, California.

Arrieta DE, VM Nihart, JD Henderson, SA McCurdy, LJ Lefkowitz and BW Wilson. A Conversion Factor Between Two Cholinesterase Assays and Its Application in Establishing Normal Range for Human RBC AChE. Presented at the 44th Annual Meeting of the Society of Toxicology; March 6-10, 2005; New Orleans, Louisiana. The Toxicologist, Vol 84, Number 5-1, Abstract No. 1278.

Wilson BW, JD Henderson, DE Arrieta, VM Nihart, MA O'Malley, SA McCurdy and LJ Lefkowitz. Monitoring Cholinesterases: Bench to Application. Presented at 25th Annual Meeting of the Southeast Pharmacology Society; November 4-5, 2004; Oxford, Mississippi.

Wilson BW, JD Henderson, DE Arrieta, A Ramirez, VM Nihart, MA O'Malley, SA McCurdy and LJ Lefkowitz. Standardizing Clinical Cholinesterase Monitoring for Pesticide Applicators and Chemical Terrorist Episodes. Presented at VIII International Meeting on Cholinesterases; September 26-30, 2004; Perugia, Italy.

CONCLUSIONS

The conversion values for delta pH and Test-Mate assays permit using the large CHPPM data base to establish a normal range for human RBC AChE in Test-Mate units. Whether or not the Test-Mate is used to establish a baseline for each soldier in the field, this normal range will be an important resource when no previous background measurement is available.

The Ellman assay appears to be more sensitive at higher activity levels than either the delta pH or Test-Mate assays (see the bi-phasic comparison curves in Figures 4 and 5). This may be due to the logarithmic measurement of the delta pH method which naturally compresses values as activity increases, and the use of a non-optimal wavelength to measure absorbance changes in the Test-Mate kit. Regardless, the effects seem to be similar and the direct comparison between delta pH and Test-Mate values is linear throughout the observed activity range. The significance is that the approved Test-Mate and delta pH tests have less sensitivity at near normal enzyme levels to detect slight exposures than when ChE activity has been severely compromised. In other words, our results to date indicate the Ellman assay is the better early warning sign of exposure.

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APPENDICES

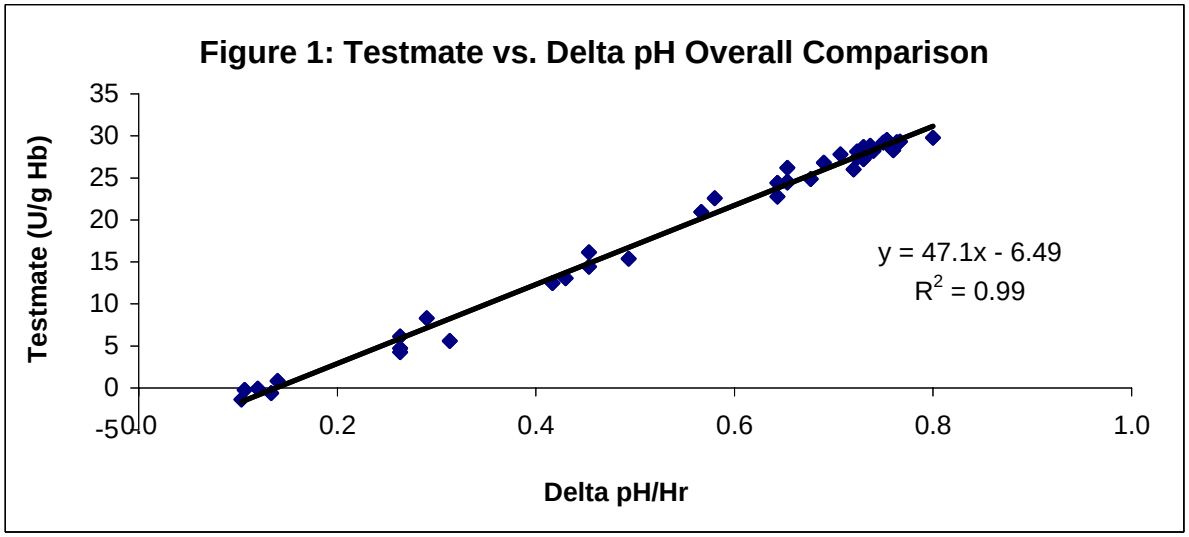
Figure 1: Testmate vs. Delta pH Overall Comparison

Figure 2: Testmate vs. Ellman Overall Comparison

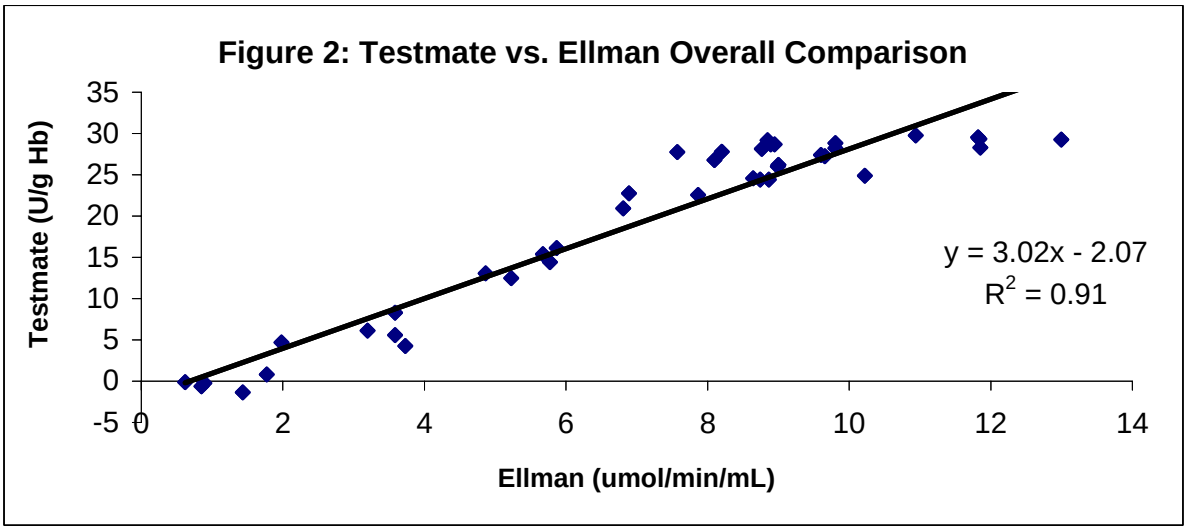
Figure 3: Delta pH vs. Ellman Overall Comparison

Figure 4: Testmate vs. Ellman Split Comparison

Figure 5: Delta pH vs. Ellman Split Comparison



Human RBCs treated with varying concentrations of DFP assayed at UCD. n = 5



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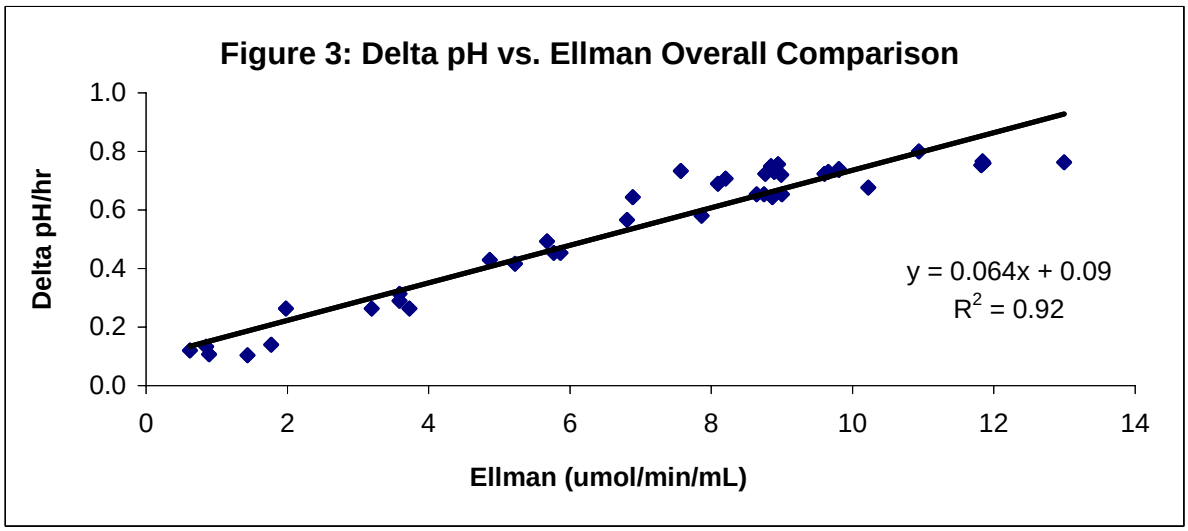

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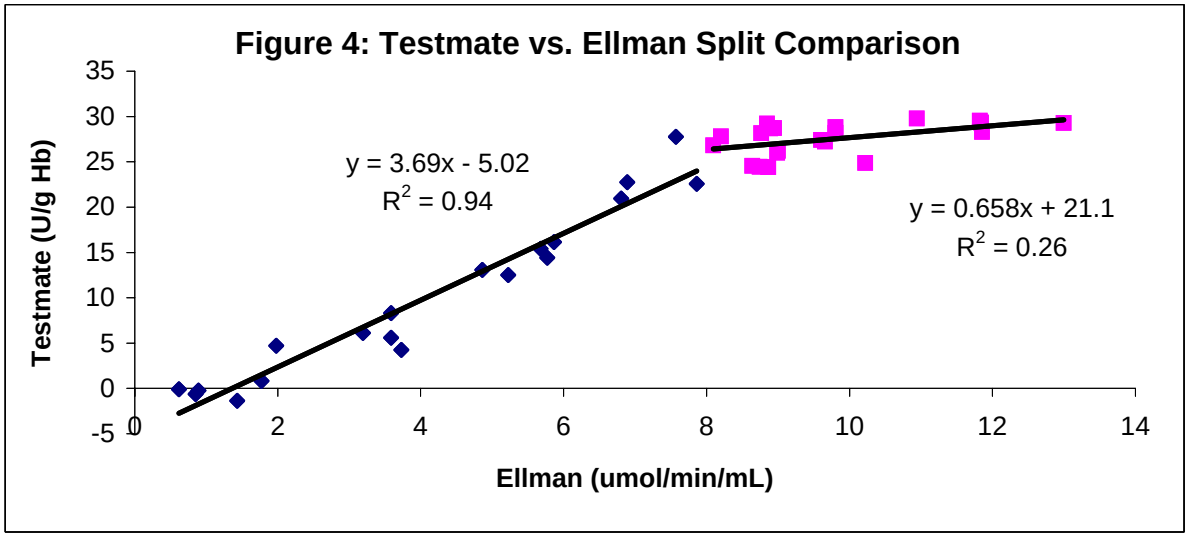

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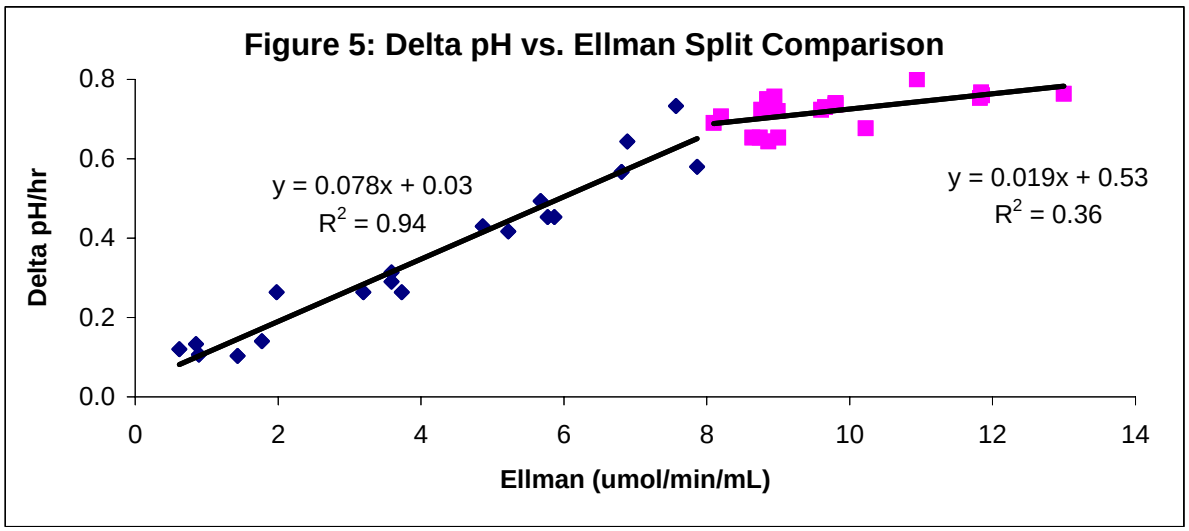

Human RBCs treated with varying concentrations of DFP assayed at UCD. n = 5